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Review

Gradient biomaterials for soft-to-hard interface tissue engineering

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ABSTRACT

Interface tissue engineering (ITE) is a rapidly developing field that aims to fabricate biological tissue alternates with the goal of repairing or regenerating the functions of diseased or damaged zones at the interface of different tissue types (also called "interface tissues"). Notable examples of the interface tissues in the human body include ligament-to-bone, tendon-to-bone and cartilage-to-bone. Engineering interface tissues is a complex process, which requires a combination of specialized biomaterials with spatially organized material composition, cell types and signaling molecules. Therefore, the use of conventional biomaterials (monophasic or composites) for ITE has certain limitations to help stimulate the tissue integration or recreating the structural organization at the junction of different tissue types. The advancement of micro- and nanotechnologies enable us to develop systems with gradients in biomaterials properties that encourage the differentiation of multiple cell phenotypes and subsequent tissue development. In this review we discuss recent developments in the fabrication of gradient biomaterials for controlling cellular behavior such as migration, differentiation and heterotypic interactions. Moreover, we give an overview of potential uses of gradient biomaterials in engineering interface tissues such as soft tissues (e.g. cartilage) to hard tissues (e.g. bone), with illustrated experimental examples. We also address fundamentals of interface tissue organization, various gradient biomaterials used in ITE, micro- and nanotechnologies employed for the fabrication of those gradients, and certain challenges that must be met in order for ITE to reach its full potential.

1. Introduction

Traditional methods for treatment of tissue loss, i.e. autografting or allografting, have limitations due to donor site shortage or immunogenicity problems [1]. Tissue engineering has the potential to overcome these clinical limitations [2]. The concept of tissue engineering uses synthetic functional components (scaffolding materials), that are cultured with appropriate cells that are usually harvested from the patient, to generate tissues that can be implanted in the patient's body [2,3].

Interface tissue engineering (ITE) is an emerging field that aims to regenerate functional tissues in order to repair or to regenerate diseased or damaged zones between different tissue types. The interfacial tissue reconstruction between soft and hard tissues (e.g. cartilage and bone) is a challenge and an indispensable question to address considering the number of people suffering from tissue or organ failure after musculoskeletal injuries, such as damage to the connection between anterior cruciate ligaments (ACL), tendons or ligaments and bones.

Tissue engineering often uses conventional biomaterials to engineer single tissues (i.e. non-interface tissues) such as skin, cartilage, bone and nerve, rather than tissue interfaces. Indeed, interface tissues in the body consist of complex structures and properties that may not be regenerated by using conventional scaffolds made of monophasic or isotropic biomaterials.

ITE (Fig. 1) focuses on the development of engineered tissue grafts capable of replacing normal function in the defective interfaces. Tissue interfaces, such as ligament-to-bone, tendon-to-bone and cartilage-to-bone, exhibit anisotropic structural properties, which gradually vary from one tissue to another. Soft tissue reconstruction methods using conventional isotropic scaffolds do not result in adequate synthetic graft integration to bones [4]. The lack of

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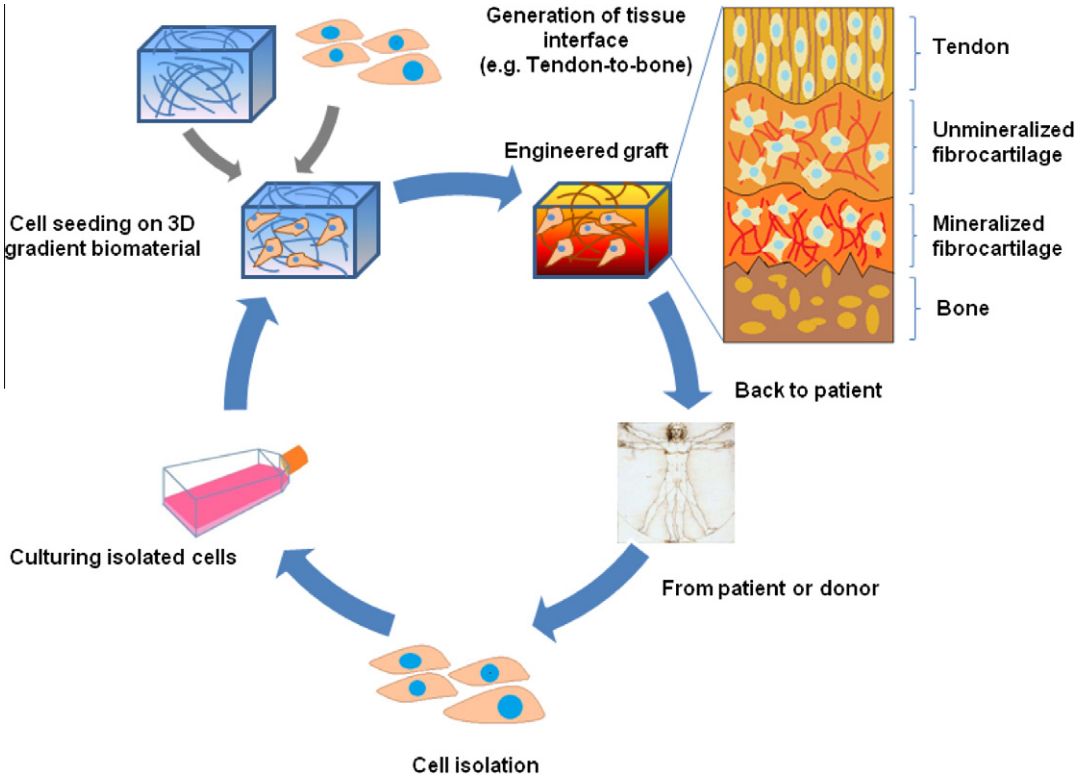

Fig. 1. Schematic illustration of the concept of interface tissue engineering.

integrating interface greatly affects the graft function. This is mainly due to the use of homogeneous biomaterials (either compositionally or structurally) to engineer tissue grafts that do not support the growth of heterogeneous cell populations that reside at the interface tissues. As a result, the implants do not fulfill their intended function, which ultimately leads to graft failures. To engineer interface tissues, a biomimetic scaffold with graded properties is therefore useful, especially since physical and chemical cues provided by the scaffold materials effect the fate of cultured cells. In a notable example, Engler et al. [5] showed that mesenchymal stem cells could differentiate into different lineages, such as neurons, myoblasts and osteoblasts, depending on the cell culture substrate's stiffness (Fig. 2). Such a gradient scaffold should provide an optimal environment to direct both heterotypic and homotypic cell–cell communications, as well as cell–matrix integrations. It should also support cellular growth and differentiation to form a graded tissue at the interface. In summary, the scaffold used to generate an interface tissue should exhibit a gradient in composition, structure and mechanical features, among other functional properties, mimicking those of the native interface zones. The advancement of micro- and nanotechnologies enable us to develop tissue scaffolds with gradient in material composition and properties that enable spatially controlled differentiation of cells and subsequent tissue development.

While previous reviews on design strategies useful in ITE have focused on parameters such as fiber stratification in engineered grafts, and multiphasing scaffolds [6–8], the potentials and challenges of biomaterials with continuous gradient in composition, structure and mechanical properties in engineering bone-integrative grafts have not been reviewed. Considering the aforementioned issues, we focus here on the design strategies of gradient biomaterials, with emphasis on microengineered hydrogel gradients and nanoengineered fibrous gradients, due to their ability in controlling the behavior of multiple cell types. Hydrogel and nanofiber scaffolding systems can be used to mimic the native extracellular matrix (ECM) viscoelastic and fibrous properties, respectively. Though they might not necessarily be ideal scaffolding systems for all kinds of ITE applications, it is promising to study their potential applications as tissue scaffolds in ITE owing to their excellent compositional, structural and other functional properties. We also discuss the biology of interface tissue organization. Finally, we conclude with current challenges and future directions in the development of gradient biomaterials towards engineering tissue interfaces.

2. Interface tissue organization

Tissues can be classified into four basic types, namely, epithelia, muscle, nerve and connective tissues [9]. Tissues can be homotypic or heterotypic in nature, meaning that they can have either homogeneous properties in terms of cell types and matrix components or heterogeneous distributions of cell types and matrix components with gradients of architecture and various other properties to fulfill their complex functions [7]. Typically, heterogeneous tissues are found at "soft-to-hard" tissue interfaces. Connective soft tissues (e.g. ligament, tendon and cartilage) connect and support other structures and organs of the body, while hard tissues (e.g. bone and teeth) primarily determine the shape of the body and provide mechanical strength required for the locomotion. Soft tissue-to-bone interfaces are ubiquitous in our body and are critical for joint motion and stabilization. These interfaces are characterized by gradual changes in properties and structural organization from one tissue to another, which allows for integration between soft and hard tissues. Some of the best studied interface tissues include ligament-to-bone, cartilage-to-bone and tendon-to-bone interfaces. The complex interface between damaged soft and hard tissues is lost during reconstructive surgery using fixation grafts, which fail to integrate into the host tissues due to their inability to create an interface tissue with graded properties and heterotypic cell culture. Therefore it is critical to develop gradient

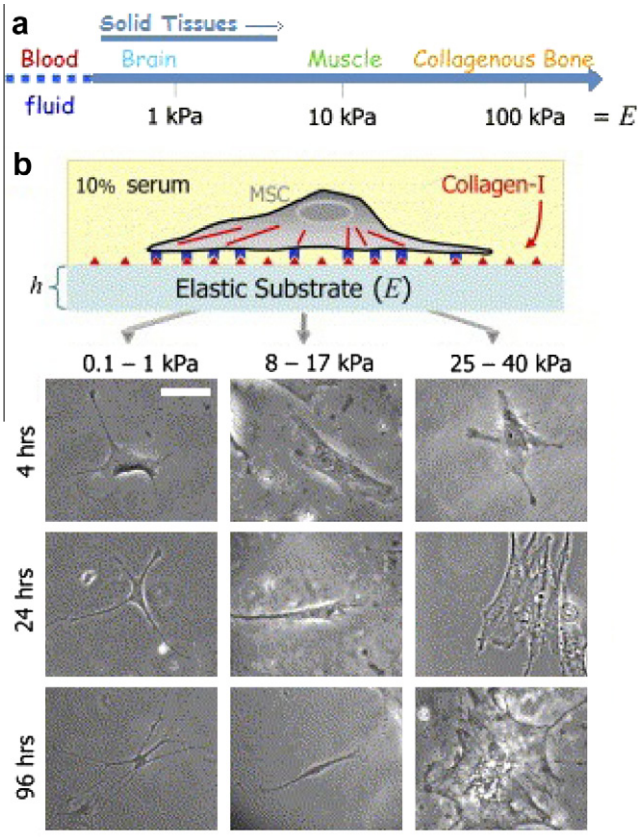

Fig. 2. Differentiation of naive MSCs directed by substrate elasticity. (A) Variations in the stiffness of example solid tissues, as measured by the elastic modulus, *E*. (B) The change in the morphology of naive MSCs grown on matrices with different elasticities, from being initially small and round into developing branched, spindle or polygonal shapes. Scale bar is 20 μm. Reprinted with permission from Ref. [5].

biomaterials to engineer grafts for full reconstruction of soft-to-hard interface tissues.

Interfaces of tissues are complex in structure, and they have a relatively small length scale (in the case of ACL, for example, it is in the order of 100 μm–1 mm, depending on the species and age factor) [10,11]. Ultrasound elastography of ACL in bovine tibio-femoral joints, as a model interface tissue, revealed that a compression-induced displacement across the insertion site was the highest at the ACL and decreased toward the bone [12]. Moffat et al. [13] showed that ligament-to-bone interface tissue tends to have more stiffness and elastic modulus from ligament to bone in a gradient manner. Further characterization of the insertion site revealed an increase in calcium and phosphate mineral contents from ligament to bone, which indicates the possibility of mineral formation in a graded fashion at the interface zones [14].

The mechanism of interface tissue regeneration and homeostasis is not completely understood. However, it is speculated that heterotypic cell communication within these complex tissues plays an important role [15–17]. In vivo transplantation of the Achilles tendon, harvested from wild-type rats, into transcondylar femoral bone tunnels of green fluorescence protein transgenic rats revealed that many host cells were detected in the graft within 1 week after implantation [16], which indicated that, in addition to osteoblasts and fibroblasts, other host cell types may be involved in fibrocartilage regeneration. Other research also supports this hypothesis [15,17]. For instance, Lim et al. [17] formed a cartilaginous tissue between a bone and a tendon graft, which was coated with stem cells, suggesting that, in addition to osteoblast and fibroblasts, precursor cells might also be involved in interface organization and regeneration. Furthermore, to study the mechanism of firbrocartilage formation, Wang et al. [15] showed that in an osteoblast–fibroblast co-culture system, the osteoblast-induced mineralization decreased while the fibroblast-induced mineralization increased, which suggests that the osteoblast–fibroblast interaction may lead to transdifferentiation of these cells at the interface, eventually forming fibrocartilage tissue. The co-culture system also promoted the expression of fibrocartilage specific markers like collagen type II (see Fig. 3B). These studies, and others, provided insights into cell–cell and ECM interactions, and their properties and effect on the formation of interface tissues, which could help understand the mechanisms involved in interface tissue organization and regeneration.

3. Development of gradient biomaterials

Biomaterials can be made of natural or synthetic origin, and are used in the construction of synthetic tissue grafts. Biomaterials can be made of a single material or be a composite of several materials. They can be modified with chemicals or biological agents, such as growth factors and adhesion peptides, in order to create suitable environments for cells to attach, proliferate and differentiate [18]. Gradient biomaterials are biomaterials with anisotropical properties, such as composition, structure, mechanics and biomolecular properties. Fig. 4A illustrates examples of continuous gradients in chemical composition, thickness and porosity [19]. Gradient biomaterials are recent additions to the biomedical field, and in particular to tissue engineering. The common design parameters of gradient scaffolds used for successful engineering of soft-to-hard interface tissues are summarized in Table 1 [20–23]. Currently, a variety of naturally derived and synthetic biomaterials are available for fabricating tissue scaffolds. These materials have been extensively reviewed elsewhere [3,24,25], so this article is limited in scope to the review of hydrogel and nanofiber gradient systems, owing to their design flexibilities and functional properties that mimic the native ECM. For instance, hydrogels exhibit hydration and viscoelastic properties close to those of native ECM, while nanofibers offer an ECM-like porous and fibrillar structure. Hydrogel and nanofibrous systems have proven to be attractive candidates for promoting three-dimensional (3-D) tissue culture in vitro, which are briefly discussed in the following sections.

3.1. Hydrogel gradient system

Hydrogels possess ECM-like viscoelastic and diffusive transport characteristics [26,27]. Their chemical and physical properties are tunable, which makes them suitable for producing tailored 3-D cellular microenvironments. However, in certain cases, hydrogels may need to be modified to support cell attachment and proliferation. Since hydrogels provide 3-D cellular microenvironments that mimic the ECM, the formation of gradients into a hydrogel system has been an attractive tool to facilitate graded tissue formation, by using the gradient hydrogel as a tissue construct. Hydrogels have been functionalized with gradients of physical and chemical cues for the purposes of high throughput screening [28,29], directed cell migration [30], axonal guidance [31] and graded cell differentiation [32]. Hydrogels with immobilized or soluble gradients of biological agents such as growth factors and adhesion peptides as well as graded physical properties such as stiffness [33] and porosity [34] have been also developed to mimic the graded features of ECM at the soft-to-hard tissue interface. The most commonly used methods of generating such gradients will be discussed in detail in Section 4.1.

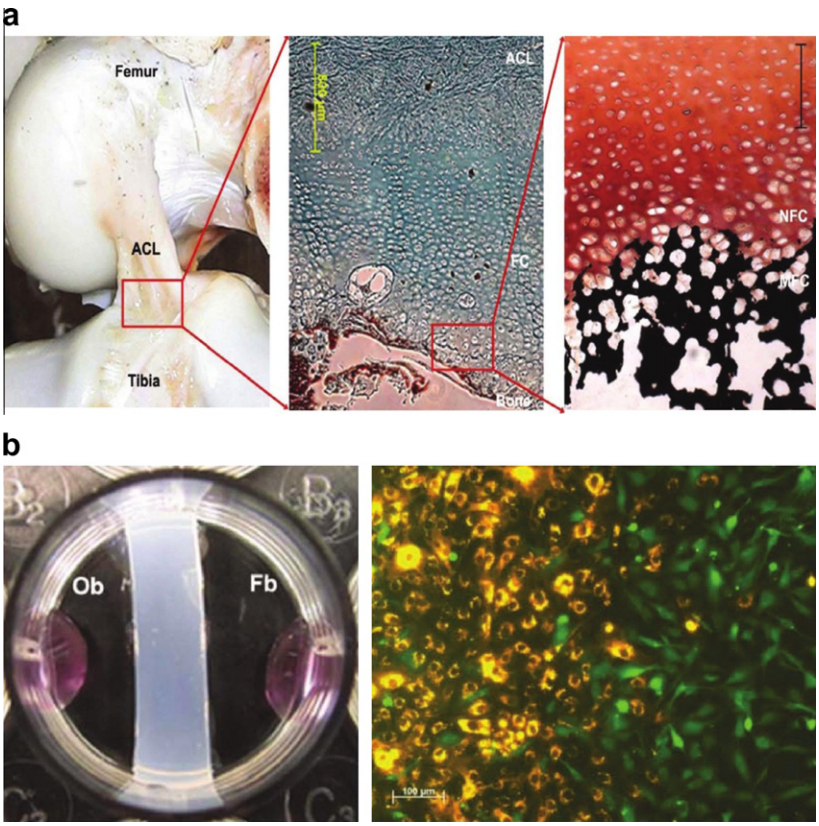

Fig. 3. (A) Anatomy and matrix organization of the ACL-to-bone insertion site. (Left) The posterior view of ACL connection to the femur and tibia through two insertion sites. (Middle) The heterogeneous tissue organization of the tibial insertion, consisting of the ACL, fibrocartilage (FC) and bone tissues. (Right) Further division of fibrocartilage interface into the nonmineralized fibrocartilage (NFC) and mineralized fibrocartilage (MFC) zones (bar = 200 μm). Adopted with permission from Ref. [7]. (B) Co-culture models to evaluate interaction of interface-relevant cells. (Left) In vitro co-culture model of fibroblasts (Fb) and osteoblasts (Ob) permit heterotypic and homotypic cell–cell interactions. (Right) Fibroblast (CFDA-SE, green) and osteoblast (CM-DiI, orange-red) distribution at day 7, bar = 100 μm. Adopted with permission from Ref. [7].

3.2. Nanofiber gradient systems

ECM contains nanofibrous proteins that provide biological and chemical functions as well as physical support for cells to grow into specific tissues. To mimic such fibrous structures for in vitro cell culture, nanofiber fabrication systems have been developed to generate polymer or composite fibers from natural or synthetic materials. These nanofibers possess a large surface area, which is favorable for cell attachment [35]. Physical and chemical properties of nanofibers can easily be tunable under appropriate conditions to facilitate cell growth and subsequent tissue development, thereby imparting gradient features into a nanofiber system is an exciting area of research. Nanofibrous scaffolds can be fabricated using methods such as self-assembly, phase separation and electrospinning [36]. The basic principles of nanofibrous scaffolds and the potential applications of electrospun nanofibers are reviewed elsewhere [3,37–39]. In this review, recent developments in generating gradient nanofiber systems are discussed (Section 4.2).

4. Micro- and nanotechnologies for generating gradient biomaterials

Table 2 [30–32,40–46] summarizes the most commonly used methods to generate gradient biomaterials. In the previous section, hydrogel gradient and nanofiber gradient systems were introduced. In the following sections, micro- and nanotechnologies employed for the fabrication of those gradient biomaterial systems are reviewed.

4.1. Formation of hydrogel gradients

Various methods have been used to generate gradient hydrogels. These include microfluidics, inkjet printing, long-range gradient makers and graded UV exposure. In the following sections, the technological advancements of these methods and their ability to generate gradients are described.

4.1.1. Microfluidics

The term "microfluidics" refers to controlling the flow of minute amounts of liquids or gases in channels with scales on the order of a few tens to hundreds of micrometers. Microfluidics is a powerful experimental tool, which generates stable concentration gradients with spatial and temporal control. Flow-based microfluidic platforms have been used to create biochemical and biomolecular gradients [47,48]. Another approach to generating gradients in microfluidic devices is to use hydrogels. These systems decrease the shear stress effect in flow-based microfluidic platforms, caused by liquids perfusion, and maintain secreted biomolecules by encapsulating cells in their microenvironment. To generate gradient hydrogels, microfluidic gradient making devices employ stepwise dilutions at multiple stages and diffusive mixing [49]. Alternative methods have also been used to make microfluidic gradient hydrogels without the use of gradient making devices. He et al. [19] developed a method to generate centimeter long gradient hydrogels. This method involved prefilling of a microchannel molded in a polydimethylsiloxane (PDMS) layer with one type of solution (often a hydrogel precursor) from the outlet of the channel, and then introducing a droplet of another solution (also a

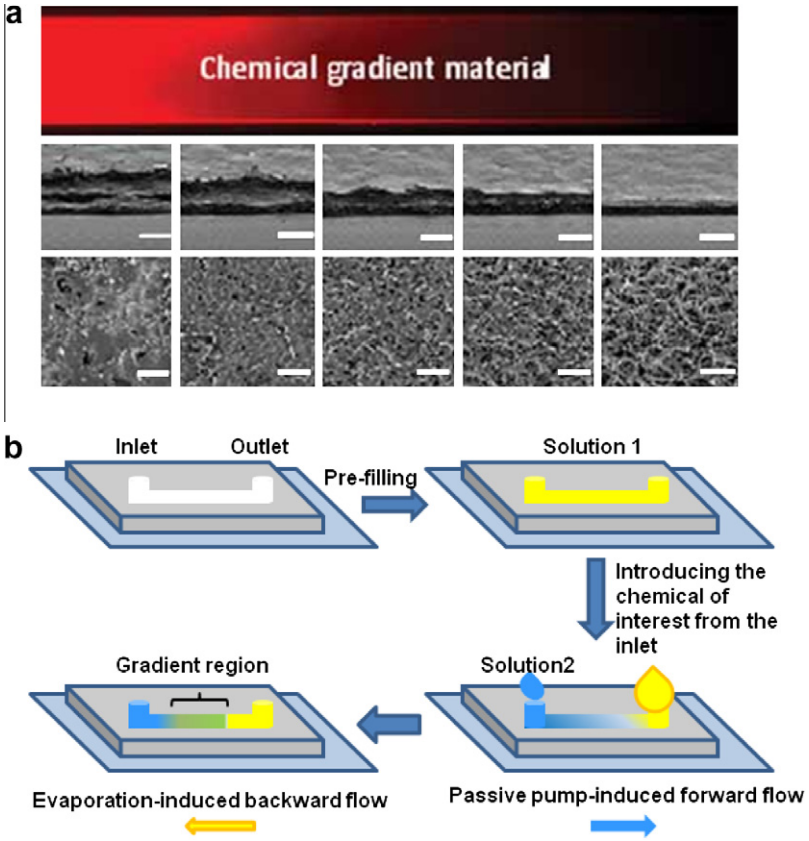

Fig. 4. (A) Examples of gradient poly(ethylene glycol)–diacrylate (PEG-DA) hydrogel fabricated in a microfluidic channel with continuous variance in chemical content, thickness (after air-drying) or porosity (after freeze-drying). Adopted with permission from Ref. [19]. (B) Gradient generation in a microfluidic channel by a passive-pumping-induced forward flow and evaporation-induced backward flow. The gradient generation device consists of a PDMS mold sealed on a glass slide. The channel is prefilled with the background solution (solution 1) from the outlet, while droplets of high-concentration chemicals of interest (solution 2) are introduced via the inlet. The solution of interest flows spontaneously into the channel by a passive-pumping-induced forward flow, and the gradient is generated by the combined effect of evaporation-induced backward flow and molecular diffusion.

Table 1
Design parameters for successful engineering of soft-to-hard interface tissues.

Properties	Remarks
Pore size and porosity	The scaffold used for ITE should have high porosity an open-cell pore structure, with a minimum pore size of 100 μM, to support heterotypic cellular interaction and calcified tissue ingrowth [20]
Architecture	The size of the tissue-engineered scaffold is an important design consideration, and should match the values for native human tisues (e.g. the size of the attachment area between human ACL and femoral bone is ~113 mm^2) [21].
Mechanical properties	Young's modulus of the scaffolds used for ITE should range between the values of soft and hard tissues: • Tibial cartilage: ~122 MPa; bone: ~1393 MPa [22]Maximum tensile strength should be higher than the level of normal human activity (e.g. for human ligament: ~67–700 N) [23].
Degradability	The scaffold designed for tissue engineering purposes should be biodegradable to be replaced with the growing tissue at a comparable pace to tissue growth
Gradient	The scaffold used for ITE should exhibit a gradient of structural and biochemical properties, mimicking the ECM of native interface tissues
Cell source	For in vitro interface tissue graft engineering, it is crucial to choose a cell with a fast growth rate to minimize the required time before implanting the graft into the patient's body

hydrogel precursor) with a high concentration of a chemical of interest from the inlet (see Fig. 4B). A passive pump-induced flow led the solution at the inlet to flow inside the channel. A backward flow induced by evaporation from the inlet of the channel then created a gradient of the chemical of interest, which was later stabilized by photopolymerization. Using this method, the authors generated a gradient concentration of adhesion peptide Arg-Gly-Asp-Ser (RGDS) along a poly(ethylene glycol) (PEG) hydrogel to test the attachment and spreading of endothelial cells. Du et al. [50] prefilled microchannels in a PDMS layer with a background hydrogel precursor polymer. The polymer of interest was then loaded at the other port and pumped back and forth at a high flow speed using a syringe pump to generate hydrogels with centimeter-long gradients of molecules, microbeads or cells within a short period (seconds to minutes). Such a cell density gradient can potentially be applied to the generation of interface tissues with heterogeneous cellular density and distribution (e.g. cartilage tissue).

4.1.2. Inkjet printing

Inkjet printing has emerged as a useful tool for creating spatially organized materials for various biosensing, tissue engineering and drug screening applications, either by direct cells positioning or by manipulating cell behavior through patterns of biomolecules, such as adhesion peptides or growth factors [51]. Basically, inkjet printing is a non-contact injection technique that

Table 2
List of the different types of gradients used in cell and tissue engineering.

Gradient type	Materials used	Applications	References
Composition	PLGA nanofiber/hydroxyapatite (HAp), collagen/HAp	Gradient mineralization of scaffolds for interface tissue engineering	[40,41]
Porosity	Agarose/gelatin hydrogel, polyacrylamide hydrogel	Microfluidic electrophoresis, bone scaffolding	[42,43]
Mechanical properties	PLGA nanofiber, agarose gel, polyacrylamide gel	Manipulating cell migration, differentiation, tendon-to-bone interface tissue engineering	[40,44,45]
Soluble molecules	Poly(2-hydroxyethylmethacrylate) microporous gel, polyacrylamide-based hydrogel	Manipulating cell attachment, migration, proliferation, differentiation, axonal guidance, tissue engineering	[31,32]
Immobilized molecules	PEG hydrogel, agarose hydrogel	Manipulating cell adhesion, alignment, migration, neurite extension, tissue engineering	[30,46]

converts digital pattern information onto a substrate, using ink drops. It can be applied to print cells, biomolecules and hydrogels on substrates. Since an inkjet printer can easily control the spatial injection of materials, it can be used for generating gradient patterns. Ilkhanizadeh et al. [32], for example, explored the potential of inkjet printing of extrinsic factors onto hydrogel-coated glass slides in directing neural stem cell differentiation. The authors generated bioprinted gradients of fibroblast growth factor, ciliary neurotrophic factor (CNTF) or fetal bovine serum on a polyacrylamide-based hydrogel-coated microscope slide. The slides with gradients of those biomolecules were used to culture neural stem cells (NSCs), and the response of the NSCs to each gradient was characterized by assessing neural differentiation. The authors reported graded differentiation of NSCs specifically on gradient CNTF-printed hydrogels. In another study, Phillippi et al. [52] generated a gradient of printed bone morphogenic protein-2 (BMP-2) on fibrin films, and studied the differentiation of mouse muscle-derived stem cells in the presence of printed gradients.

These experimental examples and others suggest that inkjet printing technology has the potential to be a widely used technique to control cell behavior in a spatially and temporally organized manner. However, there are currently a number of limitations with this technique, such as clogging of the print heads and the inability to print gradients of materials within a 3-D hydrogel. Furthermore, even though cell-laden hydrogels can be printed by using inkjet printers [53], it is currently difficult to generate large-scale tissues fabricated completely by a printing approach. Further advancement is therefore needed for this technique to realize its full potential in 3-D tissue scaffolding systems.

4.1.3. Molecular diffusion

Molecular diffusion between a source and a sink is a simple method of creating gradients. In this method, hydrogels are exposed to sources of concentrated molecules, which diffuse through them, generating a molecular concentration gradient along the hydrogel. This method has been used to generate gradients of proteins [54–56]. For instance, to study neuronal response to a gradient of laminin-1, Dodla and Bellamkonda [54] placed an agarose gel with entrapped chicken dorsal root ganglion neurons (DRG) between two sources of cell culture media. One culture medium compartment contained a high concentration of laminin-1 and the crosslinker sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (SANPAH) conjugate, laminin-1-SANPAH, while the other culture medium compartment contained a low concentration of laminin-1-SANPAH. This resulted in the gradient of laminin-1 concentration along the hydrogel, and a two times higher extension rate of DRG neurite in the anisotropic scaffolds, compared with isotropic scaffolds of LN-1. In the same context, Yamamoto et al. [56] adopted the concept of gradient incorporation of carboxyl functional groups to polyacrylamide hydrogels followed by uniform exposure of the template to collagen type I. Carboxyl group gradients in polyacrylamide hydrogels were generated by a diffusion-controlled hydrolysis of amide groups, which was performed by the generation of a sodium hydroxide gradient in the hydrogel in a side-by-side diffusion chamber at 52 °C. Attachment of L929 fibroblasts on the polyacrylamide hydrogels with a collagen gradient was studied using this system.

4.1.4. Long-range gradient maker

The aforementioned methods of generating gradient hydrogel are particularly suitable for making short-range gradients limited within a few milimeters. However, the use of long-range gradients is essential for specific cell studies that will lead to quantification of cell–material interactions. A series of commercial gradient makers have been used to make linear gradient hydrogels over 10 cm in length. These gradient makers consist of two chambers for pouring hydrogel precursors, containing high and low concentrations of the target material. The gradient makers have been used extensively to generate linear gradients of growth factors [30,31], adhesion peptide [57] and hydrogel precursor solution concentration that would result in the gradient in hydrogel stiffness [58]. Those experimental examples indicate that gradient hydrogels can be used to control directed cell alignment, migration and differentiation. For example, DeLong et al. [30] used this type of gradient maker to generate PEG-based hydrogels with immobilized gradient concentrations of basic fibroblast growth factor (bFGF) to study the migration of human aortic smooth muscle cells (HASMCs). Furthermore, they immobilized a concentration gradient of RGDS adhesion peptides in PEG-based hydrogels by using the same gradient maker and achieved the differential attachment of human dermal fibroblasts on these substrates.

4.1.5. Photopolymerization/photodegradation

An important step in generating hydrogel gradients is the stabilization of the concentration gradients of the prepolymer by chemical, ultraviolet and temperature crosslinking. In this regard, the exposure time and strength of the hydrogel prepolymer to the UV source plays an important role in the degree of crosslinking and thus the stiffness of the crosslinked hydrogel. In addition to its role in crosslinking, UV exposure of hydrogel prepolymers in a graded manner has been used as a technique to generate hydrogels with stiffness gradients. For this purpose, gradient grayscale masks have been used to expose prepolymers of methacrylated hyaluronic acid (HA) to gradients of UV light, resulting in the formation of hydrogels with elastic modulus gradients [59]. Human mesenchymal stem cells (hMSCs) cultured on those gels with gradient mechanical properties exhibited graded spreading and proliferation dependent on the local stiffness. Similarly, graded exposure of cell-laden photodegradable PEG hydrogels to UV resulted in a stiffness gradient along the scaffold, which led to a graded cellular differentiation [60].

4.2. Formation of nanofiber gradients

Nanofiber scaffolding is an emerging technology for interface tissue engineering that makes it possible to mimic the natural

fibrous ECM of a connective tissue and to fabricate fibrous scaffolding materials with a high surface-to-volume-ratio, thereby allowing cells to attach and migrate into them. Different methods of nanofiber gradient fabrications are discussed in this section.

4.2.1. Electrospinning-based method

Electrospinning is a simple, cost-effective and versatile technique that essentially employs electrostatic forces to produce polymer fibers, ranging in diameter from a few microns down to tens of nanometers. Nanofibers have been investigated as a scaffolding material for bone [61,62], meniscus [63], annulus [64] and ligament [65,66] tissue engineering owing to their high aspect ratio, surface area, permeability, porosity and tunable mechanical reliability [36,67–72], which are needed for effective cell growth in an ECM-mimicking environment. Moreover, fiber orientation (i.e. alignment) can be optimized during fabrication [36,73], to match the functional properties of the targeted tissue. Electrospun nanofibers are also suitable for surface modifications by bioactive agents to enhance their cellular compatibility. More specifically, aligned nanofibers with collagen usage can mimic bone tissue if coupled with mineralization. Such scaffolds can encourage attachment, differentiation, collagen synthesis and mineralizations of stem cells [74,75]. Shi et al. [76] fabricated polymethylglutarimide nanofibers with a fibronectin gradient by placing the electrospun nanofiber matrix vertically into a chamber, which was then filled in a controlled manner from the bottom with the fibronectin solution. The authors observed a high level of NIH3T3 cells spreading and population on the region of nanofibrous scaffold with high level of fibronectin incorporation. Li et al. [41] used a similar technique to demonstrate that nanofibrous scaffolds with gradients in mineral composition had functional effects, and led to a gradient in stiffness and cell density. In another study, the same group fabricated PLGA nanofibers by using electrospinning with a change in fiber orientation from aligned to random, which was supposed to mimic the change in collagen fiber orientation at the tendon-to-bone interface, and observed a difference in actin cytoskeleton of tendon fibroblasts in aligned and random parts of the scaffold [77]. The authors further combined this finding with their previous study, focusing on the generation of mineralized gradient nanofibers, to correlate compositional and structural properties similar to tendon-to-bone interface tissue [77]. These experimental observations, and others, clearly indicate that electrospinning can be utilized as a new approach to generate gradient scaffolds with physical and chemical properties similar to the natural ECM of fibrous tissues, such as bone or soft tissue-to-bone interface zones.

4.2.2. Hybrid of extrusion/electrospinning

Recently, extrusion and electrospinning have been combined and used to generate gradient nanofiber composites. This hybrid technique involves a twin-screw extruder with fully intermeshing and co-rotating screws combined with the electrospinning process [78]. The integration between extrusion and electrospinning techniques facilitates the incorporation of various ingredients in a time-dependent way during the electrospinning process, which results in nanofibers mats with spatially graded properties. For example, Erisken et al. [79] fabricated a composite of PCL and β-tricalcium phosphate (β-TCP) nanofibers by using this hybrid twin-screw extrusion method. The authors generated a nanofiber material with a linear variation in β-TCP concentrations, ranging from 0 to 15 wt.%, by controlling the feed rates of β-TCP nanoparticles. This system was used to study the osteogenesis of preosteoblasts on a gradient composite of PCL and β-TCP. This hybrid technique of extrusion/electrospinning is still in its infancy, and further development to realize its full potential is needed. In the next section we discuss the applications of gradient biomaterials in basic cell studies and in ITE.

5. Applications of gradient biomaterials

Gradient hydrogels and nanofiber systems have been used for basic cell characteristic studies, which are necessary for ITE. Gradient biomaterials have also been used as advantageous analytical tools with applications in drug screening and cytotoxicity tests, as well as in controlling cell behavior and tissue engineering [80,81]. Here we do not discuss the applications of gradient biomaterials in drug screening, as it does not fall within the scope of this review.

5.1. Gradient biomaterials for cellular function studies

Gradient biomaterials have been used to study cell response to in vivo-mimicking physical, chemical and biological cues, which are simulated in vitro. Studying cell response to biomimetic microenvironmental signals can help engineer biomaterials that would encourage cells to behave in a certain way (e.g. graded cell differentiation). Examples of studied cellular behaviors using gradient biomaterials include cell anchorage, migration, proliferation, differentiation and outgrowth. Generation of biomaterials which can influence cellular behavior in a graded way can potentially be useful to obtain valuable insights into designing biomaterials with gradient in composition, structure, mechanical and chemical properties to support the growth of a heterotypic cell culture required for the formation of an interface tissue.

Cell migration is an essential part of morphogenesis [82], inflammation [83,84], wound healing [85] and tumor metastasis [85]. The cell movement is encouraged by the presence of a gradient of chemical or physical cues on the surface of the culture substrate. Therefore, fabrication of biomaterials with such gradient properties can be helpful in studying cell migration in response to those graded cues. In this regard, hydrogels as well as other gradient biomaterials have gained much attention. For example, DeLong et al. [30] immobilized bFGF in PEG hydrogel scaffolds, which were functionalized with cell adhesion peptides, RGDS, to make acryloyl-PEG–RGDS conjugates. The bFGF gradient hydrogel was then evaluated for its effect on HASMC migration. The results showed an increase in HASMC proliferation, cell migration and alignment toward the bFGF concentration increase. In other studies, PEG-based hydrogel systems with gradient distributions of immobilized RGDS [57] and RGD [40] peptides were used to test cellular response to the scaffolding system orientation. Cells cultured on this RGDS gradient substrate changed their morphology to align and move in the direction of increasing RGDS concentration (Fig. 5). Luhmann et al. [86] compared the fibroblast response to 2- and 3-D gradients of the 6th Ig-like domain of cell adhesion molecule L1 (TG-L1Ig6) in fibrin matrices, and concluded that cells exposed to gradients of matrix-bound TG-L1Ig6 were able to respond differently with respect to 2- and 3-D microenvironments in terms of cell orientation and length. They also suggested that cells sense the overall concentration of a guidance cue and respond to the gradient above a certain threshold concentration by changing their alignment. Other studies have indicated that neural stem cells cultured on hydrogels printed with CNTF displayed a rapid induction of markers for astrocytes (e.g. glial fibrillary acidic protein, GFAP) [32], and that NSCs cultured on a printed gradient of increasing levels of CNTF showed a linear increase in numbers of cells expressing GFAP, demonstrating a functional gradient of CNTF. Neurite extension on gradient hydrogels has also been shown to be guided up the embedded gradients of NGF and laminin-1 [54].

In addition to gradients of immobilized biomolecules, a few studies have explored the effect of soluble biomolecular gradients on cell response. 3-D-hydrogel matrices containing soluble

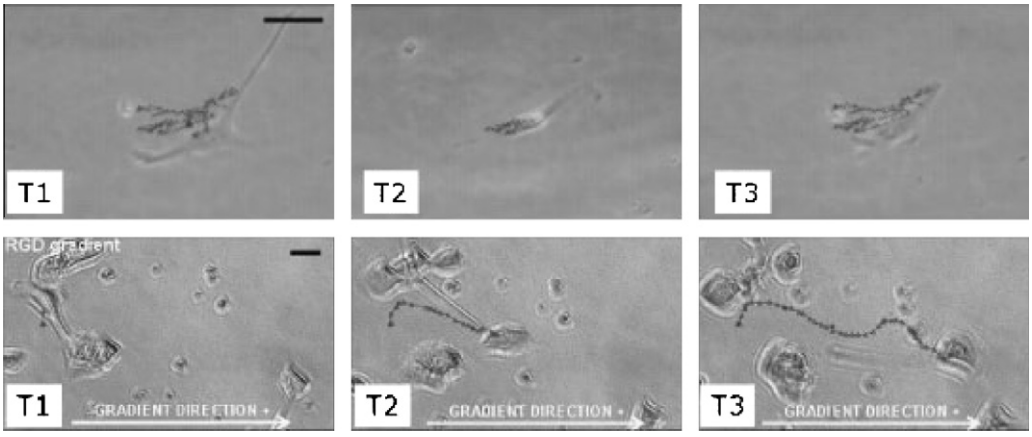

Fig. 5. Cell migration on gradient hydrogel: movie frames indicating the trajectory (black line) of mouse fibroblasts NIH-3T3 cells migrating on a PEG hydrogel with uniform distribution of RGD (top panel) and on a hydrogel with an RGD gradient (bottom panel). Bar 10 μm. Reprinted with permission from Ref. [40].

gradients of peptides have been developed to manipulate cell behavior. To study fibroblast migration, Knapp et al. [87] used a two-chamber system containing fibrin or collagen hydrogel matrices separated by a Teflon plate. One of the hydrogels contained the soluble peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) from fibronectin. After removal of the Teflon plate, these peptides diffused into the non-peptide-containing hydrogel. Gradients of soluble GRGDSP formed in the second hydrogel and were stable for 24 h. Fibroblasts embedded in the 3-D matrices changed their alignment, and migrated towards the high concentration of the soluble peptides [87].

In addition to chemical gradients that trigger cell migration (chemotaxis), mechanical properties of cell culture substrate can also lead to cell movement (mechanotaxis). Lo et al. [46] formed an acrylamide hydrogel containing a concentration gradient of bis-acrylamide cross-linker, which resulted in a hydrogel with a gradient in elastic modulus ranging from 140 to 300 kdyn cm^{-2}. Fibroblasts seeded on these gels moved toward the stiffer regions of the gels. Similarly, Wong et al. [33] observed that vascular smooth muscle cells seeded on polyacrylamide hydrogels with 1 cm long gradients in elastic modulus ranging from 5 to 35 kPa migrated to the stiffer regions of the hydrogels 24 h after seeding. Cell migration also depends on the absolute value of the elastic modulus. Studies have revealed that fibroblasts migrated in a random direction on a 100 μm long styrenated gelatin gradient with elastic modulus ranging from 200 to 400 kPa, while in the case of an elastic modulus of 10–90 or 50–350 kPa they migrated toward the higher elastic modulus area [88]. Cell movement to stiffer regions was also observed when macrophages were seeded on a 5 cm long PEGDA gradient with elastic modulus ranging from 3 to 100 kPa [64].

These experimental studies clearly suggest that cells can be manipulated by controlling the spatial presentation of physical, chemical and biological cues in an engineered material.

5.2. Gradient biomaterials for interface tissue engineering

Engineering interface tissues is a complex task, which involves the use of multiphase biomaterials allowing integration at the defective sites and promoting the development of functional tissues at the interfaces. The use of suitable cell culture substrates with the appropriate gradient in composition, structure, mechanics and biomolecular properties are indispensible to regeneration of tissue interfaces. The idea of using gradient biomaterials was initiated by the knowledge gained about the interface structure–properties relationships, and by the findings on the role of cellular interactions in interface regeneration.

Multiphased scaffolds in which every phase offers specific compositional properties have been used to develop co-culture systems of heterotypic cell populations, which are often found at the interface tissues. Spalazzi et al. [89] designed a triphasic scaffold for the regeneration of the ACL-to-bone interface in vitro, and in vivo in a rat model [90]. Fibroblasts and osteoblasts cultured on the two opposite sides of the triphasic scaffold migrated to its blank middle phase, and considerably increased matrix production. The triphasic scaffold maintained a heterotypic yet continuously integrated culture of cells, though the authors did not observe any fibrocartilage-like tissue formation within the interface between osteoblast and fibroblast co-culture.

It is hypothesized that by generating biomaterials with continuous gradients in composition, structure, mechanical and biomolecular properties a finer system could be achieved to mimic the ECM of interfaces, which would provide a more smooth integration of heterogeneous cell types. To this end, Chatterjee et al. [58] showed that material properties (gel stiffness) of scaffolds can induce cell differentiation in 3-D cultures, where PEG hydrogels with a concentration gradient of prepolymer solution yielded hydrogel scaffolds with a gradient of compressive modulus. The gradients functioned as a screening system for determining an optimum substrate modulus for osteoblastic differentiation and inducing significant mineralization. In addition to biomaterial property modification, such as composition, structure, mechanics and chemical properties, gene therapy has also been adopted as a new approach to generate tendon-to-bone interface. Phillips et al. [91] transfected fibroblasts cultured in a 3-D collagen hydrogel with a gradient concentration of Runx2/Cbfa1 transcription factor encoding gene. The gradient expression of this transcription factor induced a gradient in fibroblast differentiation into osteoblasts, as well as a gradient of matrix components expression. Soluble factors gradients embedded in hydrogels have also been used to engineer cartilage–bone tissue interfaces. In an interesting study, gradient distributions of microspheres with recombinant rhBMP-2 and insulin-like growth factor (rhIGF-I) in silk scaffolds were generated to study the differentiation of hMSCs into cartilage-to-bone interface [92]. hMSCs cultured on silk scaffolds exhibited osteogenic and chondrogenic differentiation along the concentration gradients of rhBMP-2 and rhBMP-2/rhIGF-I.

Nanofibers have also been investigated for generation of gradient biomaterials for ITE due to their ability to mimic the collagen matrix of the native interface tissue. Nanofiber scaffolds provide suitable porous architecture required to support soft and calcified

tissue ingrowth [20,93,94]. The use of nanofiber for tissue engineering has been well documented [61–66]. However, there is limited data available in the literature on the usage of nanofibers for the development of interface tissues. Erisken et al. [79] fabricated polycaprolactone nanofibers with a gradient incorporation of tricalcium phosphate using a hybrid twin-screw extrusion/electrospinning process, which was discussed in Section 4.2.2. MC3T3-E1 cells cultured on these nanofibers synthesized ECM proteins such as collagen differentially in accordance with the increase in the level of scaffold mineralization, as seen in a typical cartilage–bone interface. In the same context, Li et al. [41] generated a linear gradient of calcium phosphates on a nonwoven mat of electrospun nanofibers. The gradient in mineral content resulted in a gradient in the stiffness of the scaffold and further influenced attachment of MC3T3-E1 cells. The same group further developed nanofiber scaffolds with heterogeneous fiber orientation to mimic the structure of tendon-to-bone insertion sites [77]. Cells on the aligned fibers part of the scaffold showed a cytoskeleton with large actin filaments aligned toward fibers, while cells seeded on the random portion showed a disorganized actin cytoskeleton. Furthermore, the expression of collagen type I, which is the most abundant protein in tendon, was also observed. Therefore cells seeded across the scaffold were capable of expressing the tissue-specific type of collagen (type I) and producing the appropriate ECM for tendon repair. These experimental studies, in addition to others, clearly indicate that there is a great potential of gradient biomaterial for use in engineering interface tissues.

6. Concluding remarks

The structure complexity of heterotypic interface tissues demands a more sophisticated design of cellular constructs to meet the requirements of native cellular microenvironments with complex gradient features at the zonal interfaces. ITE aims to develop techniques to engineer heterogeneous constructs for use as tissue engineered grafts at the tissue interfaces, which fail to heal spontaneously. Biomaterials with gradients in compositional, structural and functional properties have proven to be a promising tool for engineering interface tissues.

The experimental examples summarized in this review represent some of the developments of gradient biomaterials, as heterotypic tissue constructs, from a variety of micro- and nanoscale approaches. Of particular interest, hydrogel and nanofiber gradient systems seem to be promising scaffolding systems, owing to their design flexibilities and functional properties, and the analogy to native ECM. These biomaterials can be utilized with complex gradient features to mimic the graded signals of the native cellular microenvironment. One of the most critical aspects of engineered scaffolds intended for use as a soft–hard interface tissue substitute is the ability to impart the gradient features functionally fit to the defect site and compensate for the mechanical stresses of the bone defective site. In addition, with the translation of the described methods into clinical practice, the interface scaffold needs to be adapted to the current reconstruction grafts, meaning that the scaffold with gradient properties should be well adapted to multicellular culture conditions. For example, the scaffold should be loaded with primary fibroblasts, osteoblasts and chondrocytes, harvested from the donor site. The resulting scaffold with a triculture of cells will consist of a bone-integrative side (i.e. cultured with osteoblasts) and a soft tissue-integrative side (i.e. cultured with fibroblasts/chondrocytes). Such a scaffold should be inserted into the bone defect site from the bone-integrative side, while the soft tissue-integrative part of it remains within the joint cavity and encasing the soft tissue. The stability of this system can be further increased with biodegradable screws for mechanical fixation of the graft to the body tissues. In an optimal condition, the combination of suitable compositional, structural, mechanical and biochemical properties within the scaffold should result in the creation of a completely mineralized tissue in the bone defect and a fibrocartilage tissue integrated into the host soft tissue.

The field of ITE is still in its infancy, and faces significant difficulties and challenges. Gradient biomaterials with compositional variations must support divergence in the evolving tissue interface structure to completely integrate at the host soft-to-hard tissue zone. There is also a need for developing strategies to promote vascularization and innervations within the engineered tissue graft. Importance should be given to increasing our understanding of the structure–function relationship and the detailed compositional mechanical properties of interface tissues, the basic principles governing the structural organization at the interfaces, the reasons for the failure of current interface tissue grafts and the way to improve methods of assembling multiple cell types into 3-D biomaterials at different length scales. To achieve this, future research will have to enable the development of biomaterials with more complex gradient features, which would consist of multiphase physical, chemical and biological cues to encourage culture of multiple cell types capable of integrating at the soft–hard tissue interfaces. In this regard, fabricating 3-D hydrogels with high porosity levels and improved mechanical properties through developing novel composites will provide a biomimicking microenvironment suitable for cellular proliferation, ingrowth and differentiation, while meeting the mechanical requirements of the insertion site.

Further challenges remain to be addressed to make clinical translation of gradient biomaterials suitable for emergency applications. For this purpose, methods for long-term aseptic storage of those biomaterials while maintaining their key characteristics need to be developed to minimize the waiting time required before transplantation of the engineered graft in patient's body.

Micro- and nanoscale techniques are versatile tools for the development of such gradient biomaterials and could be utilized to design a new generation of engineered grafts for use in ITE. Other techniques, such as gene therapy, could also be integrated to generate genetically modified cellular constructs with a gradient load of certain genetic information that could activate a series of autocrine and paracrine cell signaling, as observed in the native tissues. Further optimization and standardization of gradient slopes need to be done to direct cell behavior in a desired way, both in vitro and in vivo, with great challenges and also great expectations ahead.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2, 3 and 4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.01.011.

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